

Characterization of Transducing Bacteriophages Identified in Some Multi-Antibiotic Resistant Pathogenic Bacteria.

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Abstract

Proteus mirabilis is a member of Enterobacteriaceae family. It is a facultative anaerobic gram-negative bacilli, which is an opportunistic pathogen and is known to cause septicemia, meningitis, wound infections, burn infections and urinary tract infections. This bacteria is associated with development of renal stones in patients with urinary tract infections. Current practice of treatment of bacterial infections is the use of antibiotics. But many of the bacterial pathogens are turning resistant to antibiotic regimes which is a serious problem not only in medical sector but also in food and industrial sector as well. An alternative potential technique in the age of multi-drug resistance is the use of Phage therapy. Unlike antibiotics, phages are host specific and usually less toxic as compared to antibiotics. The aim of the study is to isolate bacteriophages specific to *Proteus mirabilis* from sewage by standard techniques. The isolated phages will be harvested, purified and stored which can later be used for therapeutic purposes.

Morganella morganii has been identified as a causative agent of opportunistic infections like diarrhea, wound infections, urinary tract infections, bacteremia, and sepsis. The aim of the study is to isolate bacteriophages specific to *Morganella morganii* from sewage by standard techniques. The isolated phages will be harvested, purified and stored which can later be used for therapeutic purposes.

Phage therapy is an alternative treatment to antibiotics that can overcome multi-drug resistant bacteria. In this study, we aimed to isolate and characterize bacteriophages against *Morganella Morganii* and *Proteus Mirabilis*. Bacteriophages O1 and S1 were isolated from concentrated wastewater collected from sewage.

Four phages of *proteus mirabilis* were isolated and three phages of *Morganella morganii* were isolated. phages were further characterized on the basis of plaque morphology, host range, serology, adsorption, latent period, thermal inactivation and pH stability.

morphological analysis with transmission electron microscopy show that *M. morganii* phages were identified as a *Myoviridae* bacteriophages while *p.mirabilis* were identified as a *podoviridae* and *myoviridae* bacteriophages.

Introduction

Morganella morganii, a motile gram-negative rod belong to the family *Enterobacteriaceae*, has low pathogenicity, but compromised patients can develop diarrhea, wound infections, urinary tract infections, bacteremia, and sepsis due to *M. morganii* (Tucci & Isenberg, 1981; Chang *et al.*, 2011). In addition, *M. morganii* is known as a major histamine producer due to its powerful histidine decarboxylase. *Morganella morganii* is responsible for histamine accumulation on food (López-Sabater *et al.*, 1996; Rodtong *et al.*, 2005), which often causes histamine poisoning (also referred to as scombroid poisoning) in decomposed fishery products (Becker *et al.*, 2001). Therefore, the inhibition of *M. morganii* growth in food is important to reduce the risk of histamine poisoning.

Bacteriophages are bacterial virus and a notable antimicrobial agent. The therapeutic potential of phages has not been an area of interest since the discovery of antibiotics. However, phages have recently been suggested as an alternative antibacterial agent to counteract the emergence of antibiotic-resistant bacteria (Kutateladze & Adamia, 2010). To date, no studies have focused on the use of phages to control *M. morganii*, although only a few studies related to *M. morganii* phages have been reported (Schmidt & Jeffries, 1974; Zhu *et al.*, 2010). In this study, we describe the novel *M. morganii* phage o1 isolated from sewage, and we characterize o1 for use as an antimicrobial agent against *M. morganii*.

Proteus mirabilis, a ubiquitous Gram-negative bacterium, is most frequently isolated from the gastrointestinal tracts of humans and animals (O'Hara, Brenner & Miller, 2000; Drzewiecka, 2016). Human infections with *P. mirabilis* often occur in the eyes, mouth, and intestines and urinary tract infections (UTIs) (Schaffer & Pearson, 2015; Armbruster, Mobley & Pearson, 2018). *P. mirabilis* causes between 10% and 44% of catheter-associated urinary tract infections (CAUTIs), which are among the world's most abundant nosocomial infections (Schaffer & Pearson, 2015; Miloet *et al.*, 2017). Increasing rates of antimicrobial resistance in clinical *P. mirabilis* strains is a grave concern for nosocomial *P. mirabilis* infection severity (Luzzaro *et al.*, 2001; Wang *et al.*, 2014; Girlich *et al.*, 2020).

Phages have been investigated as a treatment method for *Proteus*-based CAUTIs and associated catheter-blockages, for which established control strategies are lacking (Milo *et al.*, 2017; Maszewska *et al.*, 2018; Ujmajuridze *et al.*, 2018; Gomaa *et al.*, 2019). Byproducts of *P. mirabilis* biofilms in CAUTI cases lead to the production of crystalline aggregates which, in combination with biofilm growth, can dangerously obstruct the flow of a catheterized individual's urine (Schaffer & Pearson, 2015; Milo *et al.*, 2017).

Multiple studies have demonstrated the capacity for phages to inhibit the formation of *P. mirabilis* biofilms on catheter surfaces, which merits the further investigation and classification of *P. mirabilis* phages (Milo *et al.*, 2017; Maszewska *et al.*, 2018; Ujmajuridze *et al.*, 2018; Gomaa *et al.*, 2019). The few characterized *Proteus*-specific phages are widely variable in morphology, genome sequence length, host range, and gene content (Prozesky, Klerk & Coetzee, 1965; Alves *et al.*, 2019). In this study, we describe the novel *p.mirabilis* phage s1 isolated from sewage, and we characterize s1 for use as an antimicrobial agent against *p.mirabilis*.

Aim

To isolate. Screen and characterize an effective phage O1 for *M.morganii* and effective phage S1 for *P.mirabilis*.

Materials and Methods

1-Isolation of *M.morganii* phages

Bacteriophages were isolated from different sewage water samples obtained from Zagazig City, Egypt by the enrichment technique (Adams, *et al.*, 1959). The sewage samples were clarified by centrifugation at 6000 *g*. for 20 min, after particle removal by paper filters, the water sample was filtrated through 0.45- μ m pore membranes. Then, a 100 mL of the filtrate was added to an equal volume of nutrient broth medium and 1 mL of log phase culture of *M.morganii* and incubated in a shaking incubator 120 rpm at 37 °C for 24 h. The culture was then centrifuged at 6000 *g*. for 10 min, treated with chloroform and the supernatant filtered through 0.45 μ m pore membranes to remove the bacterial culture, and the supernatant was used as a source of phages. then 100 μ L of a mid-exponential phase of bacterial culture was added to 3 mL semi-solid NBM and poured over solid NBM agar plates. After drying 15 ml of droplets of phage source previously prepared were spotted into the lawns and left to dry. Plates were incubated overnight at 37°C and checked for the presence of lysis zones.

2- Purification and Propagation of *M.morganii* phages

Bacteriophages were propagated and purified from single plaque isolates according to previous studies (Adams, *et al.*, 1959 ; Abdel-Haliem, M.E.F.; Askora, 2013). Phage isolates were purified by picking three successive single plaques using a sterile Pasteur pipette until homogenous plaques were obtained. Briefly, a single plaque was picked and put into 5.0 mL nutrient broth containing 100 μ L of bacterial host and then

incubated at 37 °C under shaking condition with 1200 rpm. After incubation chloroform was added and the phage-host mixture centrifuged at 6000 *g*. for 10 min and the supernatant was filtered through a sterilized 0.45-µm Millipore filter (Steradisc, Kurabo Co., Ltd. Osaka, Japan) before filtration with 0.22-µm Millipore filter. The purified phage was stored at 4 °C.

3-Determination of Host Range and Cross Infectivity of the Isolated Bacteriophages of M.morganii

The isolated phage was investigated for host range specificity and lysis efficiency. Bacterial lawns off different bacterial species were propagated on NBM plates as described before, and 10-µL droplets of phage were spotted on the lawns. The plates were incubated at 37 °C for 24 h and checked for the presence of plaques. To assess the host-range of the isolated phage, *Streptococcus aureus*, *Enterococcus gallinarum*, *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were each inoculated on double-layered agar plates containing the appropriate medium, and streak tests were performed.

4-Examination of M.morganii Phage Morphology by Electron Microscopy

Bacteriophage particles were stained with Na-phosphotungstate or uranyl acetate before observation in a Hitachi H600A electron microscope, as described previously (Abdel-Haliem, M.E.F.; Askora, **2013**). A drop of phage suspension at high titer (10^{11} PFU/mL) was placed on 200 mesh copper grids with carbon-coat formvar films, and any excess drawn off by absorption to filter paper. A saturated solution of Na-phosphotungstate or uranyl acetate was then placed on the grids and the excess drawn off as before. Specimen was examined with an electron microscope in a Hitachi H600A electron microscope, Faculty of Agriculture, Mansoura University.

5-One-Step Growth Curve of M.morganii phage

One step growth experiments were performed as previously described (Pajunen, et al **2000**). Briefly, *M.morganii* strains were grown and infected with bacteriophage at MOI of 0.1 and then, allowed to adsorb for 10 min at room temperature. The mixture was centrifuged, and the pellets re-suspended in 10 mL of LB medium and incubated at 37 °C. Samples (200 µL each) were obtained at 5-min intervals and divided into two volumes of 100 µL. The first sample (without the addition of chloroform) was immediately diluted and plated for phage titration to enumerate the phage particles released from the infected bacterial cells, while chloroform was added to the other sample at a concentration of 1%) to release the intracellular phages in order to determine the eclipse period.

6-M.morganii Bacteriophage Temperature and pH Stability

The temperature stability of phage was estimated at different temperatures (40, 50, 60, 70, 80, 90, and 100 °C) over 1 h. Samples were obtained at 10 min intervals, and immediately diluted and plated for phage titration (Hammerl, et al ; **2014**). The stability of phage at different pH values (2, 4, 6, 8, 10, 12, and 13) was determined using SM phage buffer (5.8 g of NaCl, 1.2 g of MgSO₄, 50 mL of 1 M Tris-HCl pH 7.5 with 0.1 g of gelatin in 1000 mL of deionized water) and the pH adjusted by the addition of 1 M HCl or 1 M NaOH solutions. After incubation of the mixtures at 4 °C overnight, the residual phage activity was determined by plaque assay technique, as previously reported [(Pajunen, et al **2000**)].

7-Isolation and Characterization of Nucleic Acids from M.morganii Phage Particles

Phage DNA was extracted using a phenol/chloroform method (Sambrook, et al, 1989). Briefly, 200 µL of purified suspension was treated with lysis buffer (0.5% (w/v) SDS, 100 µg/mL proteinase K, 20 mM EDTA) for 1 h at 56 °C. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to remove proteinaceous material. Extraction was repeated twice, and between each step the upper aqueous phase layer was transferred into a new phage-lock gel tube and gently vortexed. The mixture was centrifuged at 10,000 *g* at 4 °C for 5 min. The supernatant was transferred to a new sterile 1.5 mL Eppendorf tube and DNA was precipitated by adding an equal volume of isopropanol and the mixture allowed to stand at 20 °C overnight. The DNA pellet was centrifuged at 10,000 *g* at 4 °C for 20 min and washed twice with 75% (v/v) ethanol and suspended in deionized water before storage at 20 °C. The DNA was digested with restriction enzymes BamHI,

EcoRI and HindIII according to the supplier's instructions (Takara Bio Inc., Kusatsu, Shiga, Japan). Conditions and buffers were chosen according to the manufacturer's instructions. The incubation times for the restriction enzymes were 2 to 4 h. The digested DNA sample was analyzed by electrophoresis at 100 V in a 1.0% (w/v) agarose gel stained with ethidium bromide using a DNA ladder as marker.

8-Adsorption assay of M.morganii Phage

Morganella morganii was incubated at 30 °C and The O1 phage was added at a multiplicity of infection (MOI) of 1, and the mixed culture was incubated at 30 °C. Every 5 min, samples were collected and centrifuged at 10 000 g for 2 min, followed by filtration using a 0.45- μ m polyvinylidene fluoride filter. The PFU of phage in the supernatant was determined using serial dilution and the double agar overlay method.

1-Isolation of P.mirabilis phage

Bacteriophages were isolated from different sewage water samples obtained from Zagazig City, Egypt by the enrichment technique (Adams, et al, 1959). The sewage samples were clarified by centrifugation at 6000 g. for 20 min, after particle removal by paper filters, the water sample was filtrated through 0.45- μ m pore membranes. Then, a 100 mL of the filtrate was added to an equal volume of nutrient broth medium and 1 mL of log phase culture of p.mirabilis and incubated in a shaking incubator 120 rpm at 37 °C for 24 h. The culture was then centrifuged at 6000 g. for 10 min, treated with chloroform and the supernatant filtered through 0.45 μ m pore membranes to remove the bacterial culture, and the supernatant was used as a source of phages . then 100 μ L of a mid-exponential phase of bacterial culture was added to 3 mL semi-solid NBM and poured over solid NBM agar plates. After drying 15 ml of droplets of phage source previously prepared were spotted into the lawns and left to dry. Plates were incubated overnight at 37°C and checked for the presence of lysis zones.

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8-Adsorption assay of P.mirabilis Phage

P.mirabilis was incubated at 30 °C and The S1 phage was added at a multiplicity of infection (MOI) of 1, and the mixed culture was incubated at 30 °C. Every 5 min, samples were collected and centrifuged at 10 000 g for 2 min, followed by filtration using a 0.45- µm polyvinylidene fluoride filter. The PFU of phage in the supernatant was determined using serial dilution and the double agar overlay method.

Results

Isolation of M.morganii Bacteriophage

Bacteriophage specific to *M.morganii* was isolated from two sewage samples collected from Zagazig , Sharkia Province, Egypt using the spot test technique on double layer agar. Of the 2 samples collected only one

showed phage activity against *M.morganii* as the host. Selection of the O1 bacteriophage was undertaken upon serial passage according to their ability to lyse a broad range of *M.morganii* isolates, generate reproducible clear zones of lysis, and their capability to replicate and produce high titers on the selected host over time. The phage was plaque purified and amplified by plate lysis in preparation for characterization.

Morphological Characterization of *M.morganii* Phages by Electron Microscopy

The morphology of O1 was characterized using TEM after staining with uranyl acetate. Based on electron micrographs (Figure 1), the O1 phage had a head measurement of 33 nm (mean SD) and a contractile tail measurement of 158 nm ($n = 8$) (Fig. 1). Therefore, it is suggested that O1 belongs to the *Myoviridae* family, a member of tailed phage, which has contractile tail consisting of a sheath and a central tube.

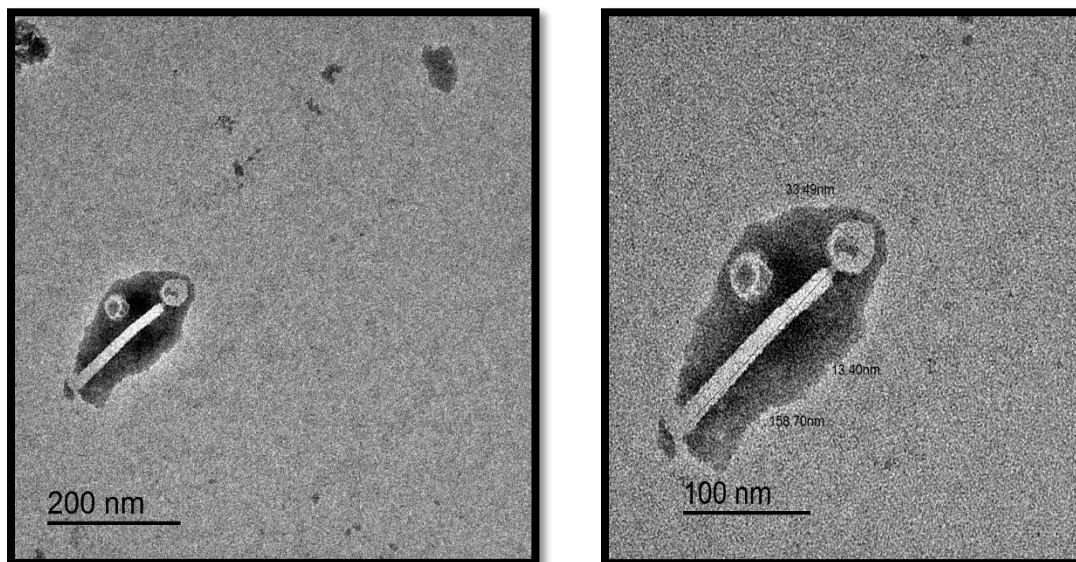


Figure 1. Transmission electron micrograph of *M.morganii* phage O1

Host range determination OF *M.morganii* phage

The host range of O1 was determined using a spot test method. One hundred microliters of each bacterial culture listed in Table 1 was added to 3.5 mL of 0.5% molten agar, and this mixture was overlaid onto a tryptic soy agar (BD) plate. Ten microliters of O1 solution was spotted onto each a agar plate. Plates were incubated at 37 °C for 6 h and were then examined for clear zones on the bacterial lawn.

Table 1. List of bacterial strains in this study and host range of O1

Bacterial host	Sensitive to O1	Description
<i>M.morganii</i>	+	Single plaques produced
<i>P. mirabilis</i>	–	No clearing
<i>Salmonella</i> Infantis	–	No clearing
<i>Proteus vulgaris</i>	–	No clearing
<i>Pseudomonas fluorescens</i>	–	no clearing
<i>Yersinia enterocolitica</i>	–	No clearing
<i>Salmonella</i> Enteritidis	–	No clearing
<i>E. coli</i>	–	No clearing
<i>K. pneumoniae</i>	–	No clearing
<i>S.aureus</i>	–	No clearing

+, clear plaque; —, no plaque.

Adsorption and one-step growth curve of O1 *M.morganella* phage

To know the O1 Growth property, we make an adsorption assay and one-step growth curve analyses. Eighty percent of O1 was adsorbed to *M. mor ganii* at 5 min. In addition, almost all of the O1 was adsorbed at 10 min (Fig.3). According to one-step growth experiment, the latent period of O1 was 10 min, the rise period was 15 min, the eclipse period was 20 min, and the burst size was 100 PFU infected cell⁻¹ (Fig. 2).

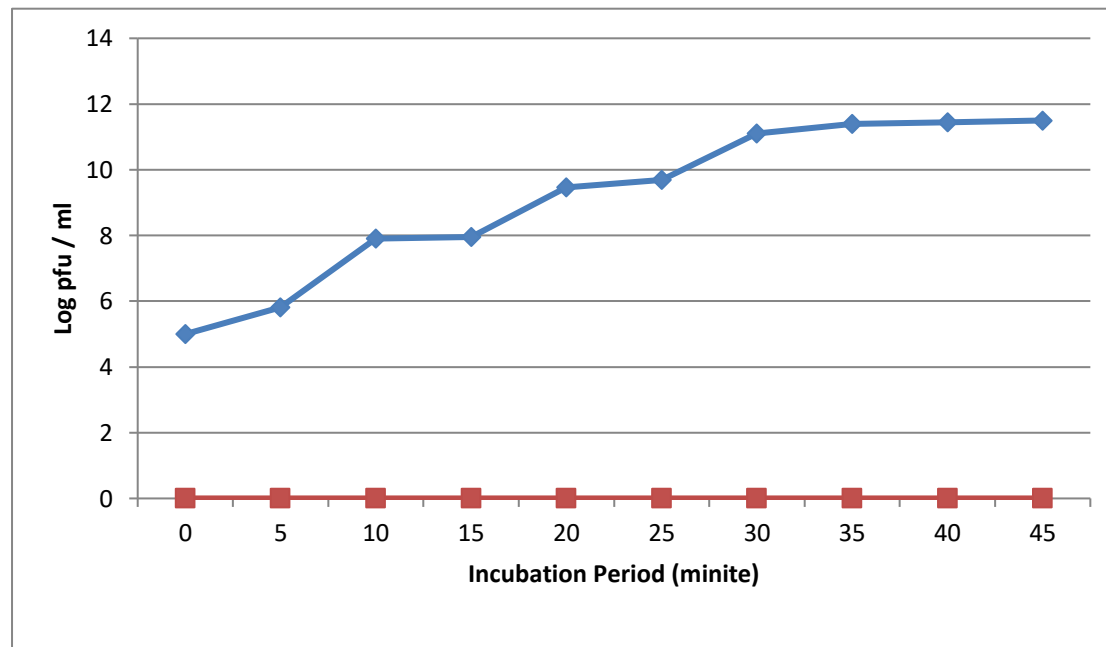


Figure 2. Single step growth curve. The blue line represents O1 phage (PFU/mL).

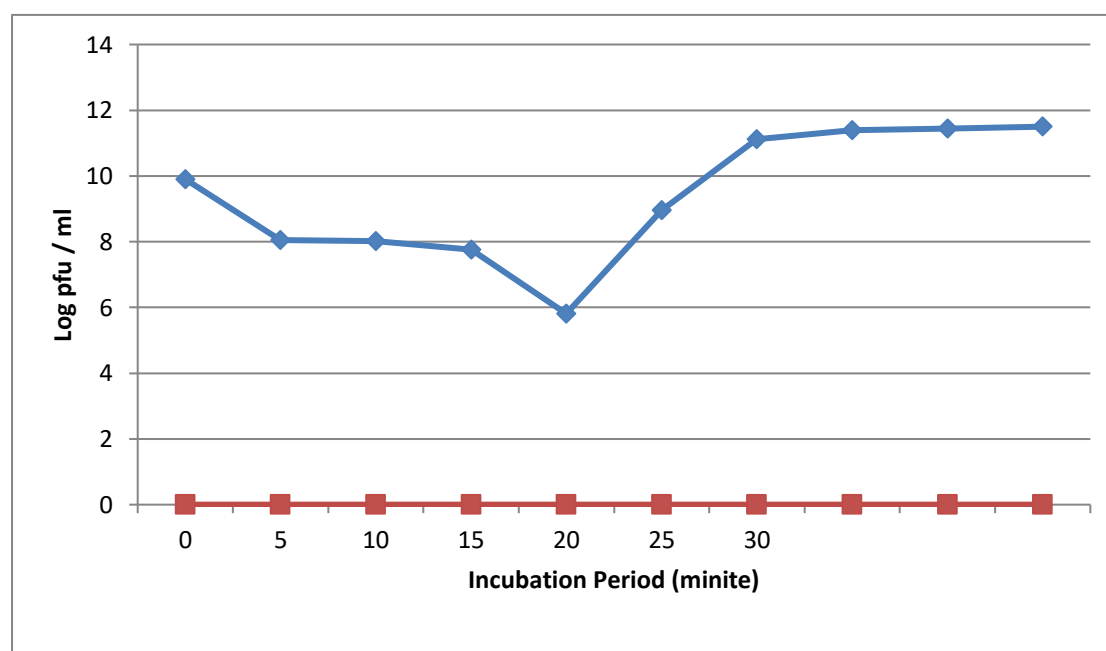


Figure 3. Adsorption rate of O1 *M.morganii*. The blue line represents O1 phage (PFU/mL).

Bacteriophage Temperature and pH Stability of O1 *M.morganii* phage

The stability of isolated phage at different temperature and pH values was investigated. The results indicated that the phage titers were stable at approximately 10^8 PFU/mL for 50 min at temperatures of 40 °C and 50 °C. At 60 °C, the phage titer decreased after 40 min to 10^6 PFU/mL. The phage titer fell to 10^4 PFU/mL after 20 min at 80 °C (Figure 5A) . The results revealed activity and stability of the phages over a broad pH range of 4 to 10 with the optimum titer at pH 8. However, postexposure to pH 2 and pH 12 the phage titer was undetectable. (Figure 5B).

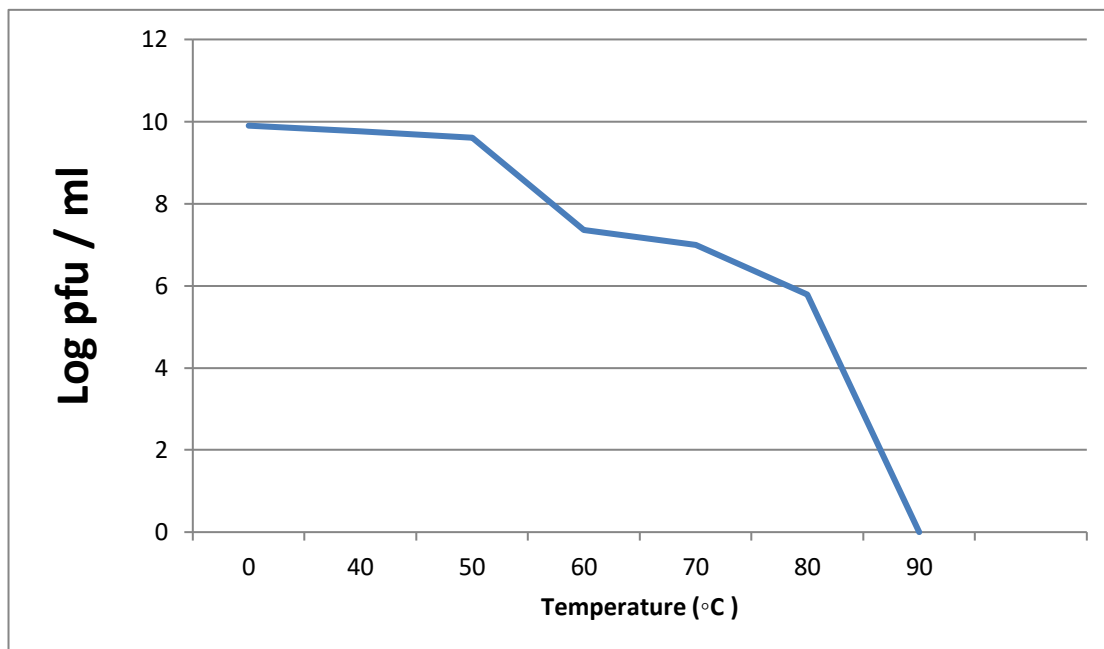


Figure 5. (A) Temperatur of *M.morganii* phage O1.

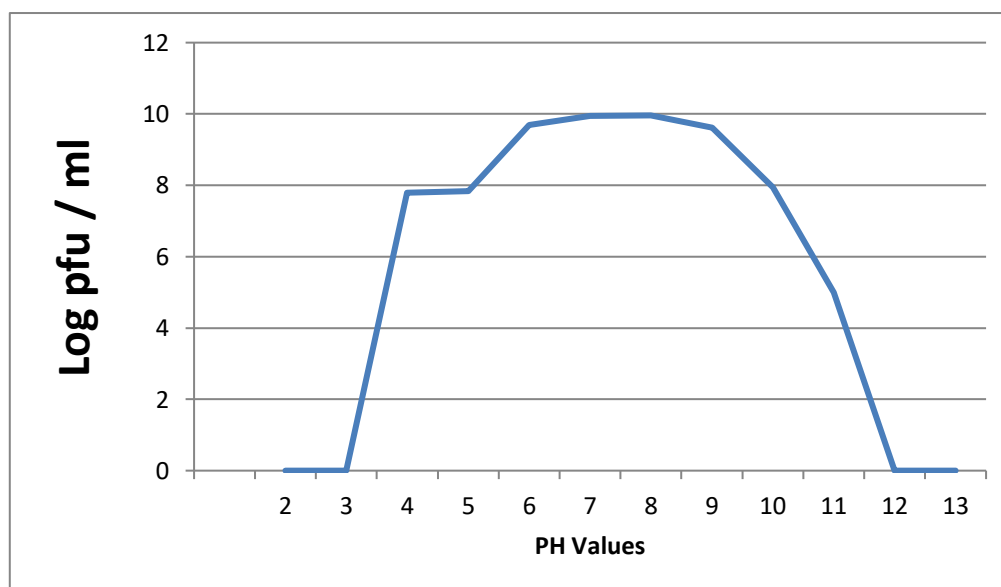


Figure 5. (B) pH stability of *M.morganii* phage O1.

Isolation of *P.mirabilis* Bacteriophage

Bacteriophage specific to *P.mirabilis* was isolated from two sewage samples collected from Zagazig , Sharkia Province, Egypt using the spot test technique on double layer agar. Of the 2 samples collected only one

showed phage activity against *P.mirabilis* as the host. Selection of the S1 bacteriophage was undertaken upon serial passage according to their ability to lyse a broad range of *P.mirabilis* isolates, generate reproducible clear zones of lysis, and their capability to replicate and produce high titers on the selected host over time. The phage was plaque purified and amplified by plate lysis in preparation for characterization.

Morphological Characterization of *P.mirabilis* Phages by Electron Microscopy

The morphology of S1 was characterized using TEM after staining with uranyl acetate. Based on electron micrographs (Figure 1), the S1 phage had a head measurement of 200 nm (mean SD) and a short tail measurement of 10 nm ($n = 8$) (Fig. 1). Therefore, it is suggested that S1 belongs to the *Podoviridae* family, a member of tailed phage, which has contractile tail consisting of a sheath and a central tube.

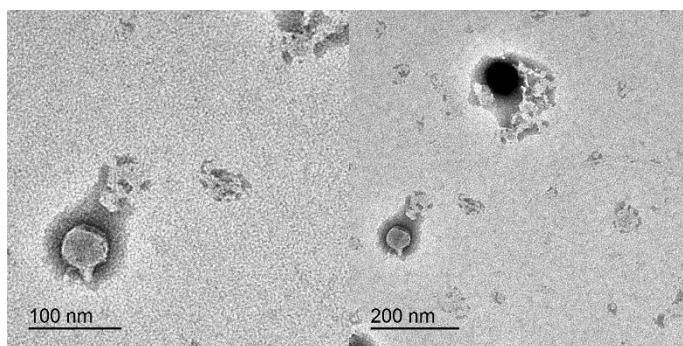


Figure 1. Transmission electron micrograph of *P.mirabilis* phage S1

Host range determination OF *P.mirabilis* phage

The host range of S1 was determined using a spot test method. One hundred microliters of each bacterial culture listed in Table 1 was added to 3.5 mL of 0.5% molten agar, and this mixture was overlaid onto a tryptic soy agar (BD) plate. Ten microliters of S1 solution was spotted onto each a agar plate. Plates were incubated at 37 °C for 6 h and were then examined for clear zones on the bacterial lawn.

Table 1. List of bacterial strains in this study and host range of S1

Bacterial host	Sensitive to S1	Description
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<i>Proteus vulgaris</i>	-	No clearing
<i>Pseudomonas fluorescens</i>	—	No clearing
<i>Yersinia enterocolitica</i>	-	No clearing
<i>Salmonella</i> Enteritidis	-	No clearing
<i>E. coli</i>	—	No clearing
<i>K. pneumoniae</i>	—	No clearing
<i>S. marcescens</i>	—	No clearing

+, clear plaque; —, no plaque.

Adsorption and one-step growth curve of S1 *P. mirabilis* phage

To know the S1 Growth property, we make an adsorption assay and one-step growth curve analyses. Eighty-five percent of S1 was adsorbed to *P.mirabilis* at 5 min. In addition, almost all of the S1 was adsorbed at 40 min. According to one-step growth experiment, the latent period of S1 was 20 min and the burst size was 90 PFU infected cell⁻¹ (Fig. 2).

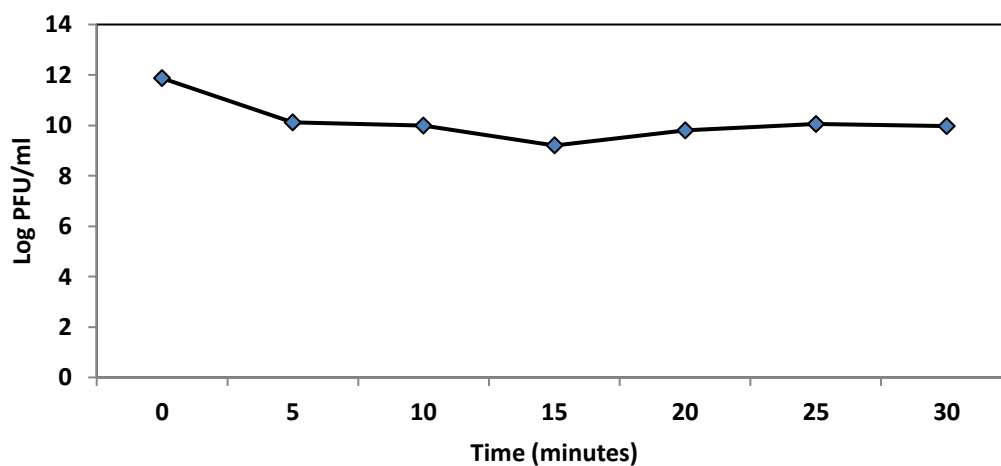


Figure 6. Adsorption rate of S1 *P. mirabilis*. The blue line represents S1 phage (PFU/mL).

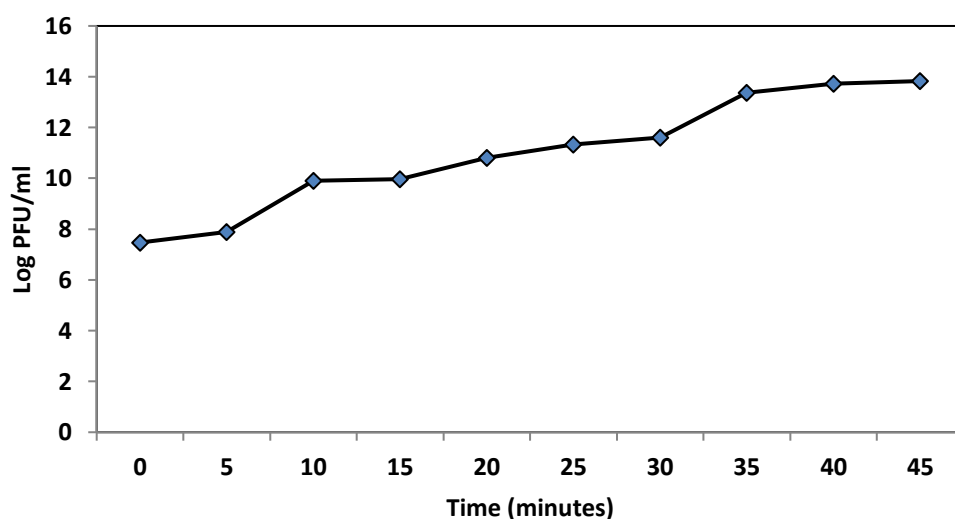


Figure 7. Single step growth curve. The blue line represents S1 phage (PFU/mL).

Bacteriophage Temperature and pH Stability of S1 *P. mirabilis* phage

The stability of isolated phage at different temperature and pH values was investigated. The results indicated that the phage titers were stable at approximately 10^{10} PFU/mL for 50 min at temperatures of 40 °C. At 50 °C, the phage titer decreased after 40 min to 10^8 PFU/mL. The phage titer fell to 10^4 PFU/mL after 20 min at 90 °C (Figure 8A). The results revealed activity and stability of the phages over a broad pH range of 4 to 11 with the optimum titer at pH 8. However, post exposure to pH 2 and pH 12 the phage titer was undetectable. (Figure 8B).

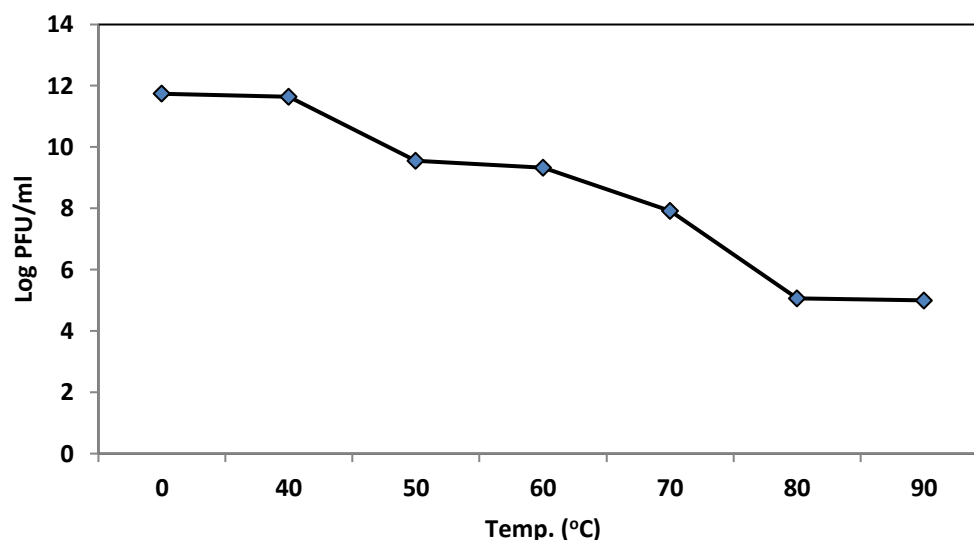


Figure 8. (A) Temperatur of *P.mirabilis* phage S1.

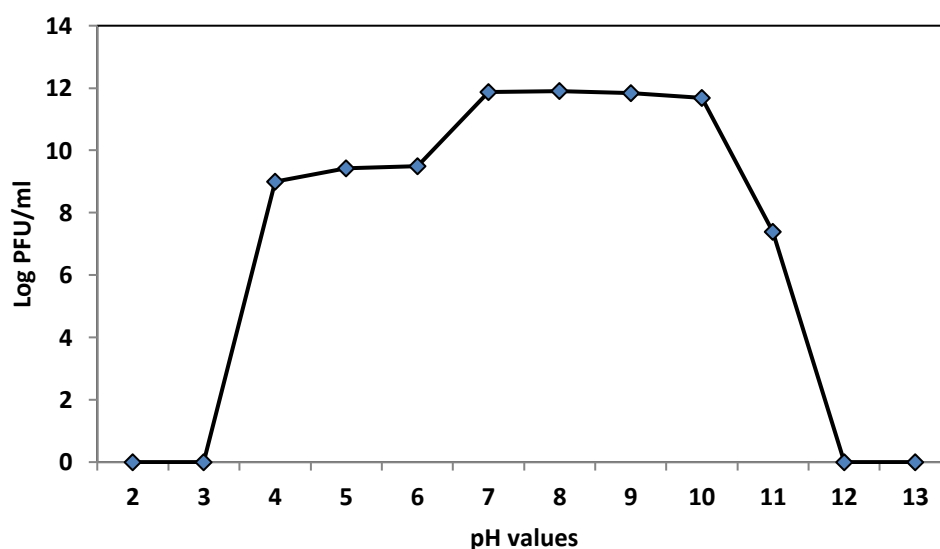


Figure 8. (B) Ph stability of *P.mirabilis* phage S1.

Discussion

Phages, which possess a different mode of action compared antibiotics, are expected to be an alternative to antibiotics (O'Flaherty *et al.*, 2009). Controlling pathogens through the use of phages has been investigated for various applications such as therapeutic drugs for human, domestic animal, plants, hatchery fish, and food additives (Carvalho *et al.*, 2010b; Gupta & Prasad, 2011; Addy *et al.*, 2012; Chibeu *et al.*, 2013; Madsen *et al.*, 2013). However, studies on the growth inhibition of *M. morganii* by lytic phages have not been reported. The aim of this study was to isolate and characterize a phage to control *M. morganii* and *P.mirabilis*.

Urinary tract infections are involved in 40% of all nosocomial infections (Saint *et al.*, 2008). Although Gram-positive bacteria, such as *Staphylococcus epidermidis* and *Enterococcus faecalis* can cause these infections, Gram-negative *Enterobacteriaceae*, are the most commonly implicated in CAUTI development (Siddiq and Darouiche, 2012). *P. mirabilis* forms crystalline biofilms within the urinary tract and is

responsible for up to 30% of all urinary tract stones (struvite) (Stickler and Morgan, 2006). Furthermore, these crystalline structures recurrently block the flow through catheters (Stickler and Feneley, 2010). As frequently observed with different bacterial species, *P. mirabilis* strains are important reservoirs of antibiotic resistant determinants (Harada et al., 2014) and, because of this, resistant phenotypes have emerged in last years (Harada et al., 2014; Wang et al., 2014). Furthermore, as in other species, *P. mirabilis* biofilms were shown to be more tolerant to several antibiotics, than their planktonic counterparts (Moryl et al., 2013).

P. mirabilis and *M. morganii* phages are isolated from sewage and were characterized. TEM analysis demonstrated that *P. mirabilis* and *M. morganii* phages belong to *Podoviridae* and the *Myoviridae* families.

Next, we examined the host range of O1 because the infection specificity of phages is generally species- or strain dependent. O1 formed plaques of the strain of *M. morganii* tested in this study, but O1 could not form plaques against other gram-positive or gram-negative bacteria, suggesting that O1 was a highly specific phage to *M. morganii*. While the host range of S1 because the infection specificity of phages is generally species- or strain dependent. S1 formed plaques of the strain of *P. mirabilis* tested in this study, but s1 could not form plaques against other gram-positive or gram-negative bacteria, suggesting that S1 was a highly specific phage to *P. mirabilis*.

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